Bacillus subtilis and *Bacillus velezensis* population dynamic and quantification of spores after inoculation on ornamental plants

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Abstract

Bacillus subtilis and *Bacillus velezensis* are used in organic agriculture as an alternative to chemical pesticides to fight against phytopathogen organisms. These Gram-positive soil-dwelling bacteria are able to resist harsh conditions and survive by differentiating into endospores. Few studies have examined how bacterial populations change on plants over time, and if they remain active or enter a dormant state. Nonetheless, these characteristics are strikingly important to determine the usage of *B. subtilis* and *B. velezensis* and their efficacy in environmental conditions. Here, we investigate the population dynamic on plants of *B. subtilis* NCIB3610 and *B. velezensis* QST713 when applied as spores on different ornamental plants. We report that on all plants studied (*Echinacea purpurea* cv. Salsa red, *Echinacea purpurea* cv. Fatal attraction and *Lavandula angustifolia* cv. Hidecote blue) spores rapidly germinated and colonized the rhizoplane, maintaining a relatively low proportion of spores in the population over time, whereas bacterial population on leaves rapidly declined. Bacteria in the surrounding soil did not germinate and persisted as spores. Taken together, these results suggest that only cells found at the rhizosphere remain metabolically active to allow the formation of a lasting relationship with the plant, making possible beneficial effects from the inoculated bacteria.

Key words: *Bacillus* — Plants — Colonization — Population — Spores

Résumé

Bacillus subtilis et *Bacillus velezensis* sont utilisées en agriculture biologique comme alternative aux pesticides chimiques. Ces bactéries Gram-positives vivant dans le sol sont capables de résister à des conditions difficiles et de survivre en se différenciant en endospores. Peu d'études ont examiné comment les bactéries persistent sur les plantes, si elles demeurent actives ou entrent en dormance. Néanmoins, ces caractéristiques sont importantes pour déterminer leur utilisation et leur efficacité dans des conditions environnementales. Nous avons étudié la dynamique de population de *B. subtilis* NCIB3610 et de *B. velezensis* QST713 lorsqu'appliquées comme spores sur différentes plantes ornementales. Nous montrons que sur toutes les plantes étudiées (*Echinacea purpurea* cv. Salsa red, *Echinacea purpurea* cv. Fatal attraction et *Lavandula angustifolia* cv. Hidecote blue) les spores ont rapidement germées et colonisées la rhizoplane, maintenant une proportion relativement faible de spores dans la population, alors que la population sur les feuilles a rapidement diminué. Les bactéries présentes dans le sol environnant n'ont pas germé et ont persisté sous forme de spores. Ces résultats suggèrent que seules les bactéries trouvées au niveau des racines restent métaboliquement actives pour permettre la formation d'une relation durable avec la plante, rendant possible les effets bénéfiques des bactéries inoculées.

Bacillus — Plantes — Colonisation — Population — Spores

Introduction

The agricultural usage of fertilizers and chemical pesticides has an important impact on both human health and ecosystem balance (Aktar et al. 2009; Nicolopoulou-Stamati et al. 2016; Carvalho 2017). Thus, there is a need to find alternative solutions with similar efficacy and reliability to help reduce the heavy use of chemical products. Plant-growth promoting rhizobacteria (PGPR) provide positive effects on plants through indirect or direct pathways (Rudrappa et al. 2008; Berg 2009; Chen et al. 2013; Chowdhury et al. 2015). These bacteria, such as members of *Azotobacter*, *Pseudomonas*, *Bacillus* and *Klebsiella* genera, can fix nitrogen, secrete growth-stimulating phytohormones and, importantly, provide resistance against a wide range of pathogens (Arkhipova et al. 2005; Ahemad and Kibret 2014).

The rhizosphere is composed of the zone directly influenced by plant roots, its exudates and mucilage, and constitutes a nutrient-rich environment hosting a complex community of microorganisms (Garbeva et al. 2004; Hartmann et al. 2008) which contrast with the surrounding soil further from the plant. Depending on the plant species, its age and the environmental conditions, root exudates composition is very dynamic and can influence the rhizosphere microbiome (Zhalnina et al. 2018). Inversely, the above-ground parts of plants are known to be a more hostile environment for microorganisms. Leaves surface, known as the phyllosphere, is particularly poor in nutrients and faces environmental stresses such as UV radiations, drastic temperature changes and variable access to moisture caused by wind and precipitations (Yang et al. 2001; Lindow and Brandl 2003; Turner 2013).

Bacillus subtilis and *Bacillus velezensis* are Gram-positive PGPR that can form endospores, allowing bacteria to survive for long periods in stressful environments (Fan et al. 2018; Hashem et al. 2019). Their capacity to withstand inhospitable environments, and thus persist over time, makes *Bacillus* spores ideal for the formulation of biofertilizers and biofungicides. However, sporulation could also limit

the duration of the bacterial beneficial activities provided to plants, since most of these activities, such as antimicrobial secretion, require an active metabolism. Currently, there is a lack of knowledge on colonization efficiency and, more importantly, on the sporulation of *Bacillus* spp. on plants in industrial growing conditions. In collaboration with a greenhouse grower, we examined the efficacy of two *Bacillus* species to colonize and to differentiate from spores into metabolically active bacteria on plants. Three ornamental plant cultivars, *Echinacea purpurea* (Purple coneflower) cv. Salsa red, *Echinacea purpurea* cv. Fatal attraction and *Lavandula angustifolia* (English lavender) cv. Hidecote blue, were chosen as models because of their market value and sensitivity to fungal pathogens. Those will thereafter be referred to as *E. purpurea* SR or FA and *L. angustifolia.*

Methods

For this study, we used *Bacillus subtilis* NCIB3610 (undomesticated strain) harboring a spectinomycin resistance cassette (Lécuyer et al. 2018) and *Bacillus velezensis* QST713 (Serenade SOIL®, Bayer), already used in Canada as a biofungicide. Bacteria were routinely grown in Luria-Bertani (LB) medium in Petri dishes (1% tryptone, 0.5% yeast extract and 0.5% NaCl) at 37 °C, and incubated in 100 mL of Difco Sporulation Medium (DSM) during 20 h at 37 °C with agitation to induce sporulation (Nicholson and Setlow 1990). Treatment at 80 °C for 20 min was used to kill vegetative cells before the spores are resuspended in sterile distilled water for inoculation. Inoculation was performed on mature plants intended for sale in garden centers. Of note, we did not observe presence of *B. subtilis* or *B. velezensis* on leaves, roots, or soil of non-inoculated *E. purpurea* FA. This result was obtained by evaluating CFU counts on a selective differential medium for *Bacillus* species (PEMBA), as described further in the text. Since all plants were in the same non-sterile soil and maintained in the same conditions, we concluded that there was no or only very few indigenous *Bacillus* on plants before we inoculated them. For inoculation, 25 mL of a *B. subtilis* spores solution at an OD_{600} = 0.75 (~$1{,}5 \times 10^7$ CFU/mL) were resuspended in deionized water and applied by

spraying onto each plant. For *B. velezensis* QST713, 10 to 30 mL (~$1.5x10^7$ CFU/mL) from the Serenade SOIL® (Bayer) were also vaporized on plants with a pump sprayer according to the usual procedures of the Plant Select company (Saint-Paul d'Abbotsford, Québec, Canada). Inoculants are applied by spraying mostly on aerial parts for timesaving. We collected samples from the phyllosphere (entire leaves), from the rhizosphere (roots parts with the soil attached) and from soil, further from the roots and their influence (from the periphery of the pot). Each sample was weighed and suspended in 5 mL of phosphate-buffered saline (PBS) for sonication treatment at 1 s start and 1 s pause for 10 s at 30% amplitude, 3 times, to remove attached bacteria and break the aggregates. To evaluate cell counts, samples were diluted and plated on selective media: LB supplemented with spectinomycin (100 µg/mL) with cycloheximide at 5 mg/L for *B. subtilis* NCIB3610 (SpecR) and PEMBA (0.1% peptone, 1% mannitol, 0.2% NaCl, 0.01% $MgSO_4$ 0.25% Na_2HPO_4, 0.025% KH_2PO_4, 1% sodium pyruvate, 0.012% bromothymol blue, 1.5% agar and 2.5% egg Yolk emulsion) with polymyxin B at 12.7 mg/L (prevents the growth of Gram-negative bacteria) supplemented with cycloheximide at 5 mg/L (inhibits growth of eukaryotes) for *B. velezensis* QST713. On this medium, QST713 forms medium-sized, flat, nearly-round with undulate margins, opaque, cream-colored, dull colonies and does not ferment mannitol nor precipitate egg yolk, allowing for a fairly precise identification. A similar phenotypical analysis was used to differentiate NCIB3610 colonies on LB spectinomycin. To evaluate the proportion of spores, the same samples were then heat-treated at 80 °C for 20 min to kill vegetative cells before inoculation on plates. We evaluated the proportion of spores in the population by dividing the number of spores by the total number of cells. Of note, few bacterial aggregates might persist through the sonication process, leading to an underestimation of the total cell count, while heat treatment allows their dissociation.

Results and discussion

Following the application of spores on above-ground parts of plants, we evaluated the population dynamics by CFU counts. At day 1, *B. subtilis* and *B. velezensis*

were found on all studied sites (leaves, soil and roots) of the three plants (Fig. 1-3). CFU counts at day 1 were generally higher on leaves, in concordance with the aerial spraying application method. However, the bacterial population on leaves never increased over time, and this for all plants, suggesting that there was no sustainable colonization on this plant site (Fig. 1A and B). On *E. purpurea* SR and *L. angustifolia* leaves, we observed a 2-fold log reduction for both *B. subtilis* and *B. velezensis* during the 15 days, while on leaves of *E. purpurea* FA populations had a smaller decrease. Bacteria in the population on leaves were mostly spores for both strains on *Echinacea* cultivars through the experiment, while on *L. angustifolia*, bacterial cells appeared to be mostly vegetative by the end of the assay (Fig. 1C and D). The decrease in total population and the large proportion spores on leaves could be explained by two phenomena. Bacteria in the phyllosphere are submitted to numerous stress factors such as nutrients depletion and fluctuation of environmental conditions (Lindow and Brandl 2003) which would explain the high number of spores on *Echinacea* cultivars and the rapid decline of the population on leaves, including on *L. angustifolia* where cells have germinated. Also, greenhouses use an aerial water-spray system which likely washes off bacteria not firmly associated with the leaf surface. Our observations that spores are predominant on plant leaves are in concordance with previous results indicating that spores represent approximately 60% of *Bacillus subtilis* UMAF6614 population after 7 days when inoculated on melon leaves (Zeriouh et al. 2014).

We also examined the presence of *Bacillus* in the soil not under the direct influence of the plant. *B. subtilis* is a soil-dwelling bacteria, but this environment contains certain constraints, especially predators, nutrients limitation and moisture availability, which do not favor its development and possess very little of the free nutrients required for its germination (Hinsinger et al. 2009; Moe 2013; Fierer 2017). Accordingly, population of both *Bacillus* species in soil remained high and stable over time but also appeared to be mostly composed of spores (Fig. 2).

Finally, we examined colonization as well as the variation in the presence of spores in the rhizosphere. For *E. purpurea* SR and *L. angustifolia,* population of both *Bacillus* species on roots remained somewhat stable through time. However, this was not the case with *E. purpurea* FA since *B. subtilis* population remained stable from day 1 to 15 but *B. velezensis* CFU count increased by 1-fold log on the root surface during the first four days (Fig. 3A and B) showing an efficient colonization. These results suggest that *B. velezensis* QST713 might be more adapted than *B. subtilis* NCIB3610 for the colonization of the rhizosphere, in the case of the studied plants. This hypothesis was further supported by the evaluation of the proportion of spores in the *Bacillus* population associated with roots. For all combinations tested, except for *B. subtilis* on *E. purpurea* FA, germination on roots was efficient since there were less than 50% of spores at most time points (Fig. 3C and D). Particularly, *B. velezensis* spores level remained very low throughout the experiment, vegetative cells sometimes reaching up to 93% of the bacterial population (Fig. 3D), which demonstrates that this population is metabolically active. The difference between the metabolic state of both strains could stem from diverse reasons, such as a better efficacy of *B. velezensis* to use nutrients sources in the root exudates or presence of germination receptors more specific to molecules secreted in the rhizosphere by these ornamental plants. Such discrepancy between bacterial strains was observed in other studies. *B. velezensis* QST713 and *B. firmus* I-1582 have different colonization efficiency when applied on corn seeds, since cell counts gradually decreased from 10^7 to 10^5 CFU/g of root for QST713 and from 10^6 to 10^4 CFU/g of root for I-1582 (Mendis et al. 2018). Of note, molecules excreted in root exudates vary between plant species, which suggests that a bacteria well adapted to the rhizosphere of one plant could be less adapted to another plant (Turner 2013).

This predominance of *B. subtilis* vegetative cells on roots of greenhouse ornamental plants contrasts with our recent observation that *B. subtilis* total number of spores rapidly increases following inoculation and germination of sporulated bacteria on *A. thaliana* roots (Charron-Lamoureux and Beauregard

2019). However, seedlings were used for the *A. thaliana* experiment, while mature plants in greenhouses were used here. The sharp difference in the amount and composition of nutrients secreted in both experimental set-ups could explain the difference in the proportions of spores. Furthermore, the *A. thaliana* study was performed in sterile hydroponic conditions, while here we used non-sterile soil, which could also contribute to the difference in metabolic activity of the bacteria. Indeed, it might be of interest to evaluate if other microorganisms from the rhizosphere can favor the vegetative state of *Bacillus* species over sporulation.

Taken together, our results show that, following application on plants, spores of both *B. subtilis* NCIB3610 and *B. velezensis* QST713 are rapidly differentiating into metabolically active cells in the rhizosphere, while those on the leaves and in the soil were more likely to stay as spores or to sporulate again after a short phase of germination. Thus, plant sites are clearly different in their impact on the proportions of dormant *Bacillus* in the population. Bacteria colonizing roots are significantly more active, strongly pointing toward the rhizosphere as being a key site for the establishment of a durable relationship between *B. subtilis* and/or *B. velezensis* and plants. These observations challenge the relevance of inoculating *Bacillus*-based biofertilizers on leaves since these spores do not appear to gain activities on this plant site. Inoculation directly into the soil, at root crown, or irrigation for the penetrance of bacteria will favor a better colonization of roots and the germination of spores into metabolically active cells. Understanding dynamics of the life cycle of *Bacillus* species used in commercial products in relation with crops for which they are used will allow us to improve how we use them. Such optimization should lead to an increase in the efficacy and reliability of various biofungicides and biopesticides used in organic agriculture.

Acknowledgments

The authors thank the company Plant Select (Saint-Paul d'Abbotsford, Québec, Canada) for the use of their facilities and the supply of plants, as well as Alain Baril and Stéphanie Théberge (Plant Select) for their support and help in this project.

They also thank Alain Lavigueur for the critical reading of the manuscript. This work was supported by an NSERC Engage grant 522757 - 17.

Conflict of interest

The authors declare no conflict of interest.

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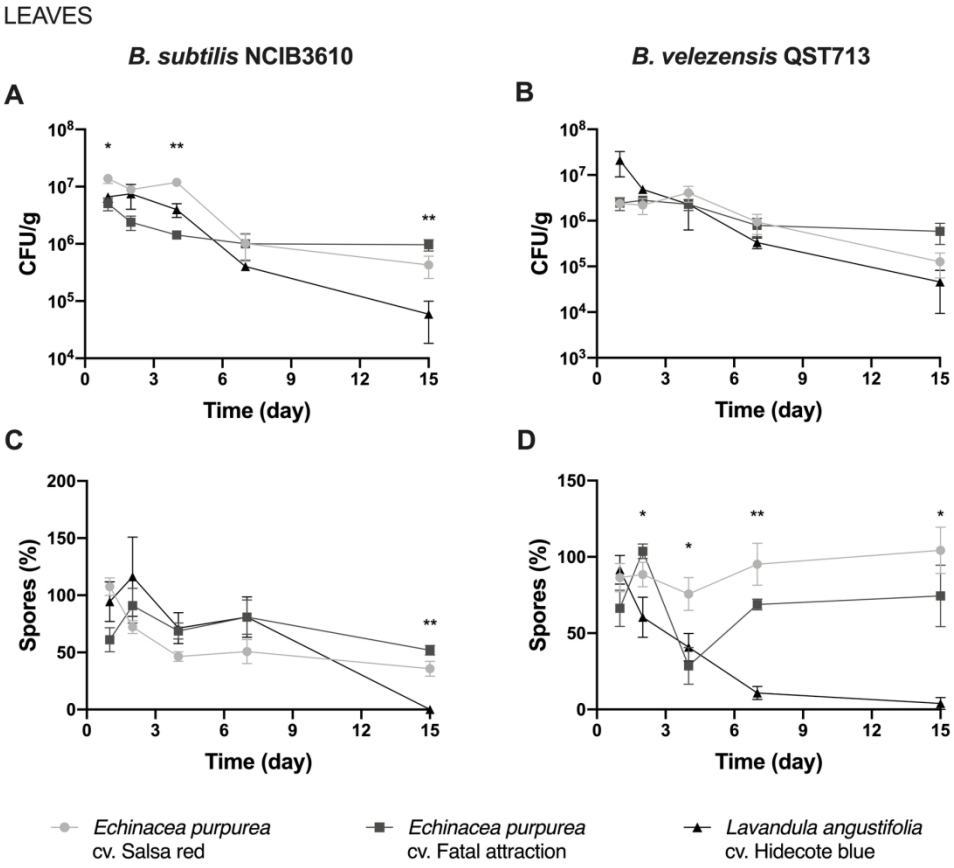
Figures legends

Fig. 1. Dynamics of population (CFU/g) and proportion of spores (%) of *B. subtilis* and *B. velezensis* on *E. purpurea* SR (gray circle), FA (dark-gray square) and *L. angustifolia* (black triangle) on leaves. **A) Total cells of *B. subtilis* on leaves.** Statistical analysis showed a significant difference between *E. purpurea* SR and the two other cultivars at day 1 and 4, and between *L. angustifolia* and the other cultivars at day 15. **B) Total cells of *B. velezensis* on leaves. C) Proportion of spores in *B. subtilis* population on leaves.** At day 15, statistical analysis demonstrated a difference between *L. angustifolia* compared to the other plant species. **D) Proportion of spores in *B. velezensis* population on leaves.** Statistical analysis revealed significant differences between *E. purpurea* FA and *L. angustifolia* at day 2, between *E. purpurea* SR and *E. purpurea* FA at day 4, between *L. angustifolia* and the two other plants at day 7, and between *L. angustifolia* and the two *Echinacea* plants at day 15. For all panels, two biological replicates composed of 4 technical replicates were combined. Error bars represent the standard error of the mean. Statistical significance was assayed using One-way ANOVA, followed by Tukey's test (* = $P < 0.05$, ** = $P < 0.01$).

Fig. 2. Dynamics of population (CFU/g) and proportion of spores (%) of *B. subtilis* and *B. velezensis* on *E. purpurea* SR (gray circle), cv. FA (dark-gray square) and *L. angustifolia* (black triangle) in soil. **A) Total cells of *B. subtilis* in the soil.** Statistical analysis showed significant differences between *E. purpurea* FA and the two other cultivars at day 1 and 4. **B) Total cells of *B. velezensis* in the soil.** At day 4, there was a statistical difference between *E. purpurea* SR and *L. angustifolia* as well as between *E. purpurea* SR compared to the other plant species at day 7. **C) Proportion of spores in *B. subtilis* population in soil.** Significant differences were observed between *E. purpurea* FA and *L. angustifolia* at day 1 and between *L. angustifolia* and *E. purpurea* SR at day 4. **D) Proportion of spores in *B. velezensis* population in the soil.** Statistical analysis revealed a significant

difference between *E. purpurea* FA and *L. angustifolia* at day 7. For all panels, two biological replicates composed of 4 technical replicates were combined. Error bars represent the standard error of the mean. Statistical significance was assayed using One-way ANOVA, followed by Tukey's test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001).

Fig. 3. Dynamics of population (CFU/g) and proportion of spores (%) of *B. subtilis* and *B. velezensis* on *E. purpurea* SR (gray circle), FA (dark-gray square) and *L. angustifolia* (black triangle) on roots. **A) Total cells of *B. subtilis* on roots.** Statistical analysis showed significant differences between all plants at day 7, and between *E. purpurea* FA, and the two other species at day 15. **B) Total cells of *B. velezensis* on roots.** At day 1, a statistical analysis demonstrated the difference between *E. purpurea* FA and *L. angustifolia*. Statistical analysis was evaluated using a t test. Letter "a" denotes a significant increase in total cells between day 1 and 2. **C) Proportion of spores in *B. subtilis* population on roots.** Significant differences were observed between *E. purpurea* FA and the other plants at day 1 and 2, and between *E. purpurea* FA and *L. angustifolia* at day 7. **D) Proportion of spores in *B. velezensis* population on roots.** Statistical analysis revealed significant differences between *E. purpurea* FA and all other plants at day 1 and at day 7, between *E. purpurea* SR and *L. angustifolia*. For all panels, two biological replicates composed of 4 technical replicates were combined. Error bars represent the standard error of the mean. Statistical significance was assayed using One-way ANOVA, followed by Tukey's test (* = P < 0.05, ** = P < 0.01, **** = P < 0.0001).



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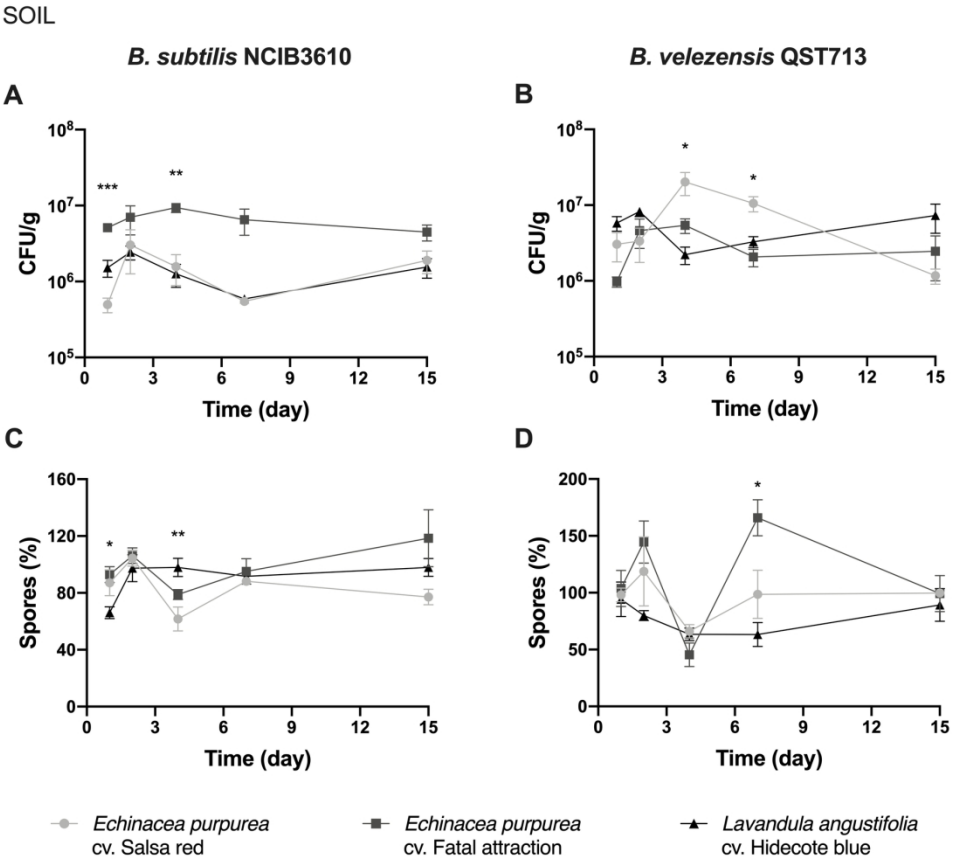

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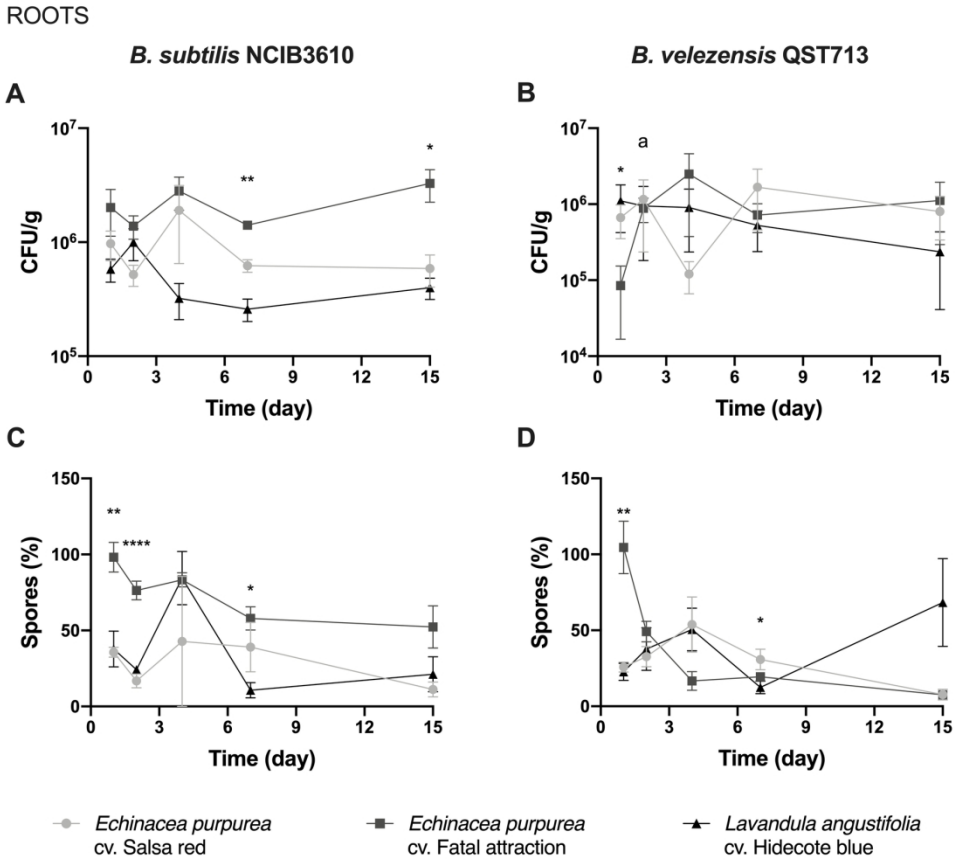

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